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(54) Title: LACTIC ACID BACTERIA FOR USE IN FERMENTED MILK PRODUCTS AND VETERINARY COMPOSITIONS

(57) Abstract

Lactic acid bacteria isolated from the gastrointestinal tract of pigs are selected for their ability to survive in a gastrointestinal environment, i.e., for their bile and acid tolerance, and for their ability to adhere to pig gastrointestinal epithelium. The thus selected bacteria may be included in a fermented milk product intended for human consumption or in a veterinary composition for preventing or treating pig gastrointestinal diseases.

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LACTIC ACID BACTERIA FOR USE IN FERMENTED MILK PRODUCTS AND VETERINARY COMPOSITIONS

FIELD OF INVENTION

The present invention relates to lactic acid bacteria isolated from the gastrointestinal tract of pigs and a fermented milk product comprising such bacteria, as well as to a powder and a veterinary composition comprising the bacterium and the use thereof in the prophylaxis or treatment of pig gastrointestinal diseases.

TECHNICAL BACKGROUND

Fermented milk products have long been known to be a beneficial component of human diet as they combine the nutritive value of milk in terms of protein content, including a favourable amino acid composition, content of fat and overall energy content (slightly less than in unfermented milk due to the conversion of lactose to lactic acid through the action of lactic acid bacteria) and the increased digestibility established for fermented milk products (Livia Alm, "The Effect of Fermentation on Nutrient in Milk and some Properties of Fermented Liquid Milk Products", *Näringsforskning 27*, 1983, pp. 2-8). The improved digestibility is obtained by precipitation of the milk protein (casein) into fine curd particles which are more easily digested by the enzymes present in the digestive tract than the large casein particles resulting from the action of gastric juice on unfermented milk. Furthermore, the fermentation process causes a significant hydrolysis of the proteins present in the milk into smaller peptides and free amino acids, and this so-called pre-digestion of the proteins makes it easier to degrade them by the digestive enzymes. Lactic acid fermentation also tends to favourably affect the utilization of calcium in the body in the form of calcium lactate which is more easily absorbed in the body.

Apart from the improved utilization of the nutrient components in milk which in itself would speak in favour of the consumption of fer-

mented milk products, the lactic acid bacteria present in the fermented milk may inhibit the growth of pathogenic or potentially pathogenic microorganisms in the intestinal tract by producing various organic acids (primarily lactic acid and, to a smaller extent, acetic, propionic and formic acid) which lower the intestinal pH so as to inhibit acid-sensitive organisms. Some lactic acid bacteria also produce antimicrobial substances known as biocines which contribute to controlling the growth of pathogens in the intestines. A high concentration of lactic acid bacteria in the intestines may also be employed prophylactically to protect against attacks from pathogenic bacteria.

Ingestion of fermented milk products may therefore be advantageous in order to maintain a good biological balance of the intestinal flora of a healthy person and possibly also to redress the imbalance in the intestinal flora occasioned by various conditions such as intestinal diseases and disorders (e.g. diarrhoea), functional disorders of the digestive tract, or the after-effects of antibiotic treatment and radiation therapy. By ingesting a fermented milk product, the normal intestinal flora may be regenerated.

Fermented milk products comprising lactic acid bacteria and alleged to exert a beneficial effect by maintaining the normal balance of the intestinal flora are commercially available and are described by, for instance, Frank V. Kosikowski in *Cheese and Fermented Milk Foods*, 2nd Ed., 1978, pp. 37-48. To the present inventors' best knowledge, however, the ability of the bacteria used in these products to survive in a gastrointestinal environment has not been adequately documented.

One object of the present invention is therefore to provide lactic acid bacteria which show advantageous properties with respect to survival in the gastrointestinal tract when included in a fermented milk product intended for human consumption.

Another object of the present invention is to provide lactic acid bacteria with useful properties for the prophylaxis or treatment of pig gastrointestinal diseases.

Gastrointestinal infections frequently occur in pigs, especially young pigs, resulting in retardation of the growth rate and even death of some of the infected pigs. Enteric diseases in pigs may be of bacterial, viral or protozoal origin and are, in the case of bacteria, initiated by a colonization of the gastrointestinal epithelium with pathogenic organisms which compete with the normal intestinal flora and, if successfully established, produce (depending on the species) exotoxins (enterotoxins) which in sufficient quantities, that is when the organism producing them has ousted the normal bacterial flora from the intestines, produce a variety of disease symptoms in the host, notably diarrhoea leading to an occasionally fatal loss of fluid from tissues and in a decreased feed consumption as well as utilization of the feed. Enteropathogenic organisms are easily transmitted from one pig to another through their presence in the faeces of infected animals present in their environment, and therefore the disease may very quickly be transmitted from the initially infected animal to the rest of the herd. Hence, gastrointestinal infections present a major source of economic loss to pig breeders.

Gastrointestinal diseases in pigs have usually been combated with antibiotics. Thus, antibiotics have been administered prophylactically to neonatal pigs or to gestating sows before farrowing. However, this procedure suffers from the disadvantage that it eliminates the natural intestinal flora, making the animals more vulnerable to invasion by opportunistic pathogens which colonize the intestines more rapidly than the natural microflora. This may, in turn, give rise to bacterial infection after the antibiotic treatment has ceased. Another frequent result of antibiotic treatment is the development of antibiotic-resistant strains of the pathogens. Furthermore, especially when therapeutic doses of antibiotics are administered to older pigs or porkers, residual amounts of the antibiotics may be present in the meat of slaughtered animals; this is undesirable since it may lead to allergic reactions in humans consuming the meat and to the development of antibiotic-resistant strains of human pathogens after exposure of these to low dosages of the antibiotics in question. For these reasons, health authorities in most countries have prohibited the presence of residual antibiotics in meat intended for human consumption.

Alternative procedures involving the use of non-pathogenic bacteria such as lactic acid bacteria have therefore been suggested as a replacement of or supplement to antibiotic treatment in case of animal, including human, gastrointestinal infections. Thus, US 3,320,130 discloses a process for preparing a medicament comprising separately culturing *L. acidophilus* and a non-pathogenic strain of *E. coli* on milk containing gastro-pyloric mucin and lysine, arginine and histidine. The separate cultures are lyophilized and mixed. Both organisms were isolated from the faeces of a healthy baby, and the medicament is, presumably, intended for human use. It is suggested that these bacteria will compete successfully with the other intestinal flora.

US 3,953,609 discloses a method of changing the digestive system bacteria in animal by orally feeding the animals live *Lactobacillus lactis* NRRL B-5628 in an amount of $1x10^{10}$ cells/kg body weight/day to reduce the number of other gastrointestinal tract bacteria. The purpose is to provide prophylaxis or treatment of colibacillosis in animals such as swine, cattle and poultry. The bacterial strain was isolated from human faeces.

US 4,314,995 discloses a method of treating infections by local administration of the *Lactobacillus* strains FRI 1946, 2779, 2780, 2781 or 2782, optionally together with an antibiotic. Among the diseases suggested for treatment in the patent by means of these bacteria are gastritis and enteritis for which applications the bacteria are preferably administered together with antibiotics. The bacteria are stated to be bile resistant, but their origin is not indicated. The bacteria appear to be intended for use in humans only.

EP 203 586 discloses the use of *Lactobacillus fermentum* ATCC 53113 or mutants thereof for treating gastrointestinal diseases in domestic animals such as pigs, cows, sheep, goats and horses. The strain is indicated to have been isolated from the gut of a healthy newborn pig.

SUMMARY OF THE INVENTION

The present inventors have now isolated probiotic lactic acid bacteria from the digestive tract of pigs which bacteria have been carefully selected for properties which are useful when the organisms are to be employed in fermented milk products for the prophylaxis or treatment of pig gastrointestinal infections.

Accordingly, the present invention relates to a lactic acid bacterium which is viable in the gastrointestinal tract of human beings and pigs, the bacterium being isolated from the gastrointestinal tract of a pig and selected according to the following criteria:

a) adhesion to pig gastrointestinal epithelial tissue after incubation of bacteria with pig epithelial cells in a ratio of 100:1 in phosphate buffered saline at 37°C for 30 minutes,

b) production of lactic acid as determined by

i) growth in MRS (Oxoid CM 359) medium, pH 6.2, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 4.0, or

ii) growth in 9.5% reconstituted skimmilk, pH 6.3-6.4, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 5.2, or

iii) growth in modified MRS medium without glucose, pH 6.2, containing equal amounts of maltose and soluble starch for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 4.5, or

iv) defined as the production of at least 10 g/l of lactic acid (D- and L-forms) when grown in MRS medium, pH 6.2, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2,

c) bile tolerance as determined by growth in MRS medium, pH 6.2, supplemented with 0.9% bile for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction comparable to the one defined in b) (i)-(iii),

d) acid tolerance as determined by a reduction in the number of colony-forming units/ml after incubation for 1 hour at 40°C and a pH of 2.5 not exceeding 3.2 log units, preferably less than 1 log unit,

e) a generation time of about 65 minutes, preferably 30-50 minutes, when grown in MRS medium at 40°C under anaerobic conditions,

or a functionally equivalent mutant thereof.

In the present context, the term "probiotic" is used to indicate cultures of non-pathogenic gastrointestinal bacteria or compositions containing such cultures which, after the ingestion of effective doses, may survive or even become established in the gastrointestinal tract and thereby preserve or enforce the function of the microflora as a barrier against the colonization of the epithelium by pathogenic organisms.

In order to exert the above-described beneficial effects both with respect to maintaining or restoring a healthy balance of the gastrointestinal microflora and with respect to inhibiting the growth of pathogenic or potentially pathogenic organisms, it is essential that the bacteria of the invention are capable of surviving the conditions prevailing in the gastrointestinal tract. The criteria according to which the bacteria of the invention have been selected have primarily been employed to achieve this overall effect. Thus, contrary to known fermented milk products allegedly producing these effects on ingestion, the lactic acid bacteria of the invention have been systematically screened for their viability under gastrointestinal conditions in pigs. Due to the well-known similarities between the human and porcine digestive systems, the fact that the bacteria of the invention have been shown to establish themselves in the intestines of pigs renders probable their ability to survive in the human gastrointestinal tract as well. When the bacteria of the invention are inge-

sted primarily to maintain a favourable balance of the intestinal microflora, survival of the bacteria in the digestive tract is the most important selection criterion as regular ingestion of a fermented milk product containing the bacteria will usually be sufficient to ensure a sufficient number of the bacteria in the intestines to provide a favourable regulation of the intestinal flora.

In connection with research leading to the present invention, it has been found that lactic acid bacteria isolated from the intestinal flora of the same animal species as the one to which it will eventually be administered for therapeutic purposes show superior properties with respect to therapeutic effect. This may, presumably, be ascribed to the fact that the bacteria have already adapted to the conditions prevailing in the intestines of the animal species in question so that they will be able to colonize and compete successfully with the other intestinal flora and establish itself in the intestines. To the best of the present inventors' knowledge, this finding has not previously been reported, and in the prior literature nobody appears to have speculated that it might be important for obtaining the desired effect, cf. EP 203 586 which although it discloses an organism isolated from a pig which is used to treat diarrhoea in pigs, also indicates that the same organism may be used for treating animals of other species.

In particular when the bacteria of the invention are intended to be used therapeutically, however, it has been found to be advantageous that they show good adhesion properties since this improves their ability to colonize the gastrointestinal mucosa and compete successfully with the pathogens or potential pathogens present in the intestines, thus obtaining a favourable balance of the gastrointestinal microflora. The adhering bacteria form a layer on the epithelium and prevent access of the pathogens to the receptors present on the epithelial cells. The bacteria preferably adhere to pig gastrointestinal tissue in a number of at least 4, preferably at least 16, most preferably at least 50, per epithelial cell. The production of lactic acid is another important selection criterion since most pathogens show a significantly decreased growth rate in the presence of acid. Bile and acid tolerance are also essential for a probiotic bacterium

to be employed successfully in the intestines, in particular the upper small intestines in order to be able to survive passage of the stomach and intestines, and possibly to colonize the epithelium in the vicinity of the biliary tract. Since most pathogens tend to multiply very quickly, it is also essential that the lactic acid bacteria employed as the probiotic organisms have a brief generation time so that they may compete with the pathogens in the intestines.

Certain lactic acid bacteria have been found to produce one or more antimicrobial agents such as hydrogen peroxide. Such substances are known to possess an inhibitory effect on a variety of microorganisms and therefore supplement the effect of pH decrease ascribable to the production of lactic acid.

Mutants of the bacteria may either occur spontaneously (as is often the case in nature) or be produced deliberately such as by treatment with a chemical mutagen such as mitomycin C, 5-bromouracil, methylmethane sulphonate, nitrogen mustard and the nitrofurans, ionizing radiation, ultraviolet radiation, or by applying recombinant DNA techniques. The term "functionally equivalent" should be taken to mean a mutant which shows similar properties to the parent strain with respect to establishing itself and competing with the pathogens present in the intestines at the time the bacteria are administered or entering the intestines at some later point.

Ingestion of a fermented milk product containing lactic acid bacteria of the invention for the purpose of regulating digestive functions or prophylactic or therapeutic administration of a lactic acid bacterium of the invention promotes the establishment of the normal, healthy microflora in the intestines thereby creating or recreating a balanced intestinal environment where enteropathogens have difficulties in establishing themselves. As a result of this, it may, in case of actual gastrointestinal infections, furthermore be advantageous to administer the lactic acid bacteria concomitantly with or subsequently to antibiotic treatment in order to restore a healthy balance of the intestinal flora. It is, however, preferred that antibiotic treatment of pig gastrointestinal tract infections be dispensed with

altogether and replaced by administration of the lactic acid bacteria of the invention.

DETAILED DISCLOSURE OF THE INVENTION

Probiotic bacteria useful for the present purpose may, in principle, be any lactic acid bacteria isolated from porcine gastrointestinal epithelium, preferably the epithelium of healthy pigs. Suitable strains may be selected after isolation by screening procedures comprising testing for adhesive properties, production of lactic acid, acid and bile tolerance and generation time as described in further detail in Examples 1-5 and 8 below. For treating pig gastrointestinal infections in particular, the bacteria may be isolated from pigs of varying ages so as to ensure the availability of strains which are able to colonize the epithelium at different stages of the pigs' lives. Specific examples of lactic acid bacterial strains which may be employed in the present invention include strains selected from the genus *Lactobacillus*, e.g. *L. acidophilus/L. gasseri* isolated from the duodenum of a suckling pig; *L. salivarius* subsp. *salivarius* isolated from the jejunum of a weaning pig; *L. acidophilus/L. gasseri* isolated from the ileum of a weaning pig; *L. crispatus* isolated from the jejunum of a suckling pig; and *L. salivarius* subsp. *salivarius* isolated from the *pars oesophagea* of a suckling pig.

Samples of thus isolated strains of these *Lactobacillus* species were deposited in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure on 22 December, 1987 in the Deutsche Sammlung von Mikroorganismen, Mascheroder Weg 1B, 3300 Braunschweig, Federal Republic of Germany, with the following accession numbers:

L. acidophilus/L. gasseri SS28	DSM 4324
L. salivarius subsp. *salivarius* SS129	DSM 4325
L. acidophilus/L. gasseri SS131	DSM 4326
L. crispatus SS151	DSM 4327
L. salivarius subsp. *salivarius* SS258	DSM 4328

It will, however, be evident to persons skilled in the art that other lactic acid bacterial strains may be isolated in a similar fashion, using the selection criteria indicated above and the procedures described in the examples, without departing from the spirit and scope of the present invention.

For use in a fermented milk product, the two *Lactobacillus salivarius* strains indicated above have been found to be particularly suitable as they not only provide the beneficial effects described above, but also result in an agreeable taste of the product.

Once isolated, the bacteria may be propagated by growing the selected strain or strains in a suitable culture medium under anaerobic conditions for a period of time sufficient to provide at least 10^6 viable bacteria/ml of medium and harvesting the resulting bacteria from the medium. The cultivation temperature will typically be about 37°C, and the pH in the range of about 5.5-6.5.

In another aspect, the present invention relates to a liquid or frozen concentrate comprising lactic acid bacteria of the invention. Although it is theoretically possible to use the culture medium containing the bacteria directly, it is generally preferred to produce a bacterial concentrate in order to obtain a higher count of bacteria for administration or inoculation. The concentrate may be prepared by, for instance, centrifugation or filtration. The concentrate may suitably comprise $1x10^4$-$1x10^{12}$ viable colony-forming units/ml. The concentrate may contain a mixture of two or more of the *Lactobacillus* strains indicated above.

In a further aspect, the present invention relates to a freeze-dried or spray-dried powder comprising a lactic acid bacterium as described above. In this case, too, it is in theory possible to dry the bacterial cultures as grown, but it is preferred to concentrate the bacteria before drying for the reason indicated above. To ensure a sufficient viability of the bacteria, it is preferred to add a stabilising or cryoprotective agent to the concentrate before drying as for instance disclosed in US 3,897,307. The resulting dried powder preferably comprises 10^4-10^{12} viable colony-forming units/g. It may be

an advantage that the powder comprises a mixture of two or more lactic acid strains such as those listed above, in order to secure a broad-spectrum activity of the powder when used as or in a preparation to be administered to pigs of varying ages.

In a still further aspect, the invention relates to a fermented milk product which comprises a lactic acid bacterium of the present invention. The product is primarily intended for human consumption, and although it is primarily ingested for its nutritive value, it may be particularly useful for dietary purposes, i.e. for its favourable influence on digestive properties ascribable to the ability of the lactic acid bacteria to inhibit the growth of pathogenic or potentially pathogenic microorganisms. The fermented milk product of the invention may contain a mixture of two or more of the *Lactobacillus* strains listed above, and preferably also contains a *Bifidobacterium* sp. such as *B. bifidum* or *B. longum*, as well as *L. acidophilus*.

The fermented milk product may be prepared by inoculating milk with a culture of the bacterium of the invention in a manner known *per se*, e.g. as described by A.Y. Tamine and R.K. Robinson in *Yoghurt Science and Technology*, 1985, preferably in the form of the liquid or frozen concentrate or freeze-dried or spray-dried powder described above. Consequently, the present invention also relates to the use of the lactic acid bacterium or a liquid or frozen concentrate or freeze-dried or spray-dried powder containing it for the production of a fermented milk product. The inoculation level is typically in the range of $1x10^5$-$1x10^8$ bacteria/ml of milk, such as $5x10^6$ bacteria/ml.

The lactic acid bacteria of the invention have been found capable of reducing the occurrence and/or severity of gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms. The term "reducing the occurrence" is understood to mean that there are fewer cases of gastrointestinal infections both among individual pigs within each herd and among herds on the same farm, relative to pigs which have not been treated with the lactic acid bacteria of the invention. The gastrointestinal tract infections which may be prevented or treated by administering the bacteria of the invention may be any infections resulting from the presence and multiplication of

enteropathogenic microorganisms in the intestines, e.g. diarrhoeas, scours, etc. Examples of enteropathogens are *E. coli* and rotavirus.

In a still further aspect, the present invention relates to a veterinary composition for the prophylaxis or treatment of gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms, the composition comprising a lactic acid bacterium as described above and an excipient or carrier. The composition should preferably comprise 10^3-10^{12} viable colony-forming units/g since in this range a sufficient number of bacteria will normally survive the passage of the pigs' stomach to colonize the intestinal epithelium in sufficient numbers to compete successfully with the pathogens or other microorganisms present.

The composition may be formulated according to conventional veterinary practice as a powder, granulate, tablet, capsule, paste, gel, drench, mixture or suspension. Solid formulations, i.e. powders, granulates, tablets and capsules, may contains fillers, e.g. sugars, sorbitol, mannitol and silicic acid; binders, e.g. cellulose derivatives such as carboxymethyl cellulose and polyvinylpyrrolidone; disintegrants, e.g. starch, sodium bicarbonate and calcium carbonate; lubricants, e.g. magnesium stearate, talc and calcium stearate. Semi-solid formulations, i.e. pastes or gels, may comprise a gelling agent such as an alginate, gelatin, carrageenan, tragacanth gum and pectin, a mineral oil such as liquid paraffin, a vegetable oil such as corn oil, sunflower oil, rape oil and grape kernel oil, as well as a thickener such as a starch, gum, gelatin, etc. Liquid formulations, i.e. drenches, mixtures and suspensions, may comprise a liquid or oily vehicle, e.g. water (favourably the drinking water of the pigs to which the composition is administered), an electrolyte solution (often given to pigs to compensate for the electrolyte loss caused by the loss of fluids from tissue which is characteristic of diarrhoea) or reconstituted sowmilk replacer. Oily vehicles comprise a mineral oil such as liquid paraffin, a vegetable oil such as corn oil, sunflower oil, rape oil, grape kernel oil, etc. The freeze-dried or spray-dried powder of the invention may be suspended in the liquid vehicle in accordance with usual practice.

The powder formulation may advantageously be the freeze-dried or spray-dried powder described above, either in itself or formulated with one or more further excipients.

The solid formulations may be provided with a coating to protect the bacteria from gastric conditions so that they will more easily survive the passage of the stomach. The coating should be one which is degradable in a gastrointestinal environment. The coating is preferably one which is soluble in the stomach (though only gradually so that the protective effect will be maintained) or in the upper part of the small intestines. Examples of suitable coatings are alginates or lipids.

The composition of the invention may also comprise a mixture of two or more lactic acid bacterial strains such as the strains listed above. Apart from the lactic acid bacteria, the composition may further comprise other active agents such as antibiotics, chemical growth promoters, or microorganisms or enzymes which have a growth promoting effect.

In a still further aspect, the present invention relates to the use of a lactic acid bacterium as defined above for preparing a veterinary composition for the prophylaxis or treatment of gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms.

It is known that pigs are most susceptible to gastrointestinal infections at three points in their lives: when they are newborn, when they are being weaned and when they have just been weaned, since their digestive system is under stress, being forced to adapt to changed feeding conditions. The composition of the invention is therefore advantageously administered to suckling or weaning pigs. However, older pigs (porkers) may also succumb to gastrointestinal diseases which may spread very quickly to an entire herd of the pigs thereby causing substantial economic loss due to death or considerable loss of weight.

The composition may be formulated in any of the ways described above, but is preferably adapted to be sprayed or sprinkled in the environ-

ment of the pigs, or is in the form of a suspension in an oily or aqueous medium such as the drinking water, an electrolyte solution or a reconstituted sowmilk replacer. When the composition is sprayed or sprinkled in the environment of the pigs, the pigs will take up the bacteria when they root about in the sty, the bacteria colonizing the intestinal epithelium and competing with enteropathogens taken up in a similar way. For weaning pigs or porkers, the composition may also advantageously be mixed with the feed. The composition is preferably the freeze-dried or spray-dried powder described above.

When the composition is intended for oral use, the amount of lactic acid bacteria per dosage of the composition is in the range of 10^4-10^{12}, preferably 10^6-10^{10}, colony-forming units. At this dosage level, the composition is suitably administered 1-3 times a day for a period of up to 14 days, the period being to some extent determined by the level of contamination in the sty or by the condition of the pigs, i.e. whether the composition is to be used therapeutically or prophylactically.

The lactic acid bacteria of the invention may optionally be administered concomitantly with or subsequent to antibiotic therapy in order to counteract the unfavourable effect of antibiotic treatment that the healthy intestinal flora is killed, thus giving pathogens ample opportunity to colonize the intestinal epithelium without being held in check by other organisms in the intestinal environment.

Alternatively, the composition of the invention may be administered to a gestating or lactating sow in an amount sufficient to provide a therapeutically effective amount of the bacteria in the environment of the sow. This form of administration therefore has the same effect as spraying or sprinkling the bacteria directly in the environment of the pigs. The amount of bacteria administered is in the range of 10^5-10^{12}, preferably 10^7-10^{11}, viable colony-forming units per dosage. At this dosage level, the bacteria are suitably administered 1-3 times a day for a period of up to 28 days, the period being to some extent determined by the level of contamination by pathogens in the sty.

The invention is described in further detail in the following examples which should not be construed as limiting in any way to the scope of the invention.

EXAMPLE 1

Isolation and characterization of *Lactobacillus* strains

Description of strains

A large number of bacteria were isolated from a number of healthy piglets and weaning pigs from several different swine herds, and after comprehensive screening as described below in Examples 1-5 and 8, the following strains were selected:

SS28 *L. acidophilus/L. gasseri*
isolated from duodenum of a suckling pig.

SS129 *L. salivarius* subsp. *salivarius*
isolated from jejunum of a weaning pig.

SS131 *L. acidophilus/L. gasseri*
isolated from ileum of a weaning pig.

SS151 *L. crispatus*
isolated from jejunum of a suckling pig.

SS258 *L. salivarius* subsp. *salivarius*
isolated from *pars oesophagea* of a suckling pig.

Fermentation pattern

The fermentation profile of the bacteria is shown in Tables 2, 2a and 3. The profiles were performed using a commercially available test system (API-50CH), Tables 2 and 2a, and using a modified MRS (Oxoid CM 359) medium and incubation method according to *Bergey's Manual of Systematic Bacteriology*, Volume 2, 1986, Table 3.

For identification, the fermentation profiles of both tests were compared to the fermentation patterns described in *Bergey's Manual* 1986. In addition to the fermentation patterns of the 22 carbohydrates from the API-50CH used for comparison with *Bergey's Manual*, eight more criteria shown in Table 2a were also taken into account in the identification, resulting in 30 criteria according to *Bergey's Manual*.

Table 2

Fermentation patterns as recorded by API-50CH

	Medium	Reaction				
		SS28	SS129	SS131	SS151	SS258
	Glycerol	-	-	-	-	-
	Erythritol	-	-	-	-	-
)	D-Arabinose	-	-	-	-	-
x)	L-Arabinose	-	-	-	-	-
x)	Ribose	-	-	-	-	-
)	D-Xylose	-	-	-	-	-
x)	L-Xylose	-	-	-	-	-
	Adonitol	-	-	-	-	-
	β Methyl-Xyloside	-	-	-	-	-
x)	Galactose	+	+	+	+	+
x)	D-Glucose	+	+	+	+	+
x)	D-Fructose	+	+	+	+	+
x)	D-Mannose	+	+	+	+	+
	L-Sorbose	-	-	-	-	-
x)	Rhamnose	-	+	-	-	+
	Dulcitol	-	-	-	-	-
	Inositol	-	-	-	-	-
x)	Mannitol	-	+	-	-	+
x)	Sorbitol	-	+	-	-	+
	α Methyl-D-Mannoside	-	-	-	-	-
	α Methyl-D-Glucoside	-	-	-	-	-
	N Acetyl Glucosamine	+	+	+	+	+
x)	Amygdaline	+	-	-	+	-
	Arbutine	+	-	-	+	-
x)	Esculine	+	-	+	+	-
x)	Salicine	+	-	-	+	-
x)	Cellobiose	+	-	+	+	-
x)	Maltose	+	+	+	+	+
x)	Lactose	+	+	+	+	+
x)	Melibiose	+	+	-	-	+
x)	Saccharose	+	+	+	+	+
x)	Trehalose	+	-	+	-	+
	Inuline	-	-	-	-	-
x)	Melezitose	-	-	-	-	-
x)	D-Raffinose	+	+	+	-	+
	Amidon	+	-	+	+	-
	Glycogene	-	-	-	-	-
	Xylitol	-	+	-	-	+
	β Gentibiose	+	-	+	+	-
	D-Turanose	-	-	-	-	-
	D-Lyxose	-	-	-	-	-
	D-Tagatose	-	-	-	-	-
	D-Fucose	-	-	-	-	-
	L-Fucose	-	-	-	-	-
	D-Arabitol	-	+	-	-	+
	L-Arabitol	-	-	-	-	-

Table 2 (continued)

x)	Gluconate	-	-	-	-	-
	2 Ceto-Gluconate	-	-	-	-	-
	5 Ceto-Gluconate	-	-	-	-	-

x) Reactions for comparison according to Bergey's Manual together with reactions from Table 2a

Table 2a

Criteria	Reaction SS28	SS129	SS131	SS151	SS258
Growth at 15°C, 5 days	-	-	-	-	-
Growth at 45°C, 5 days	+	+	+	+	+
Gas from glucose, 5 days	-	-	-	-	-
Gas from gluconate, 5 days	-	-	-	-	-
L-Lactic acid produced, g/l, * (Boehringer Mannheim Kit)	5.9	14.0	4.5	7.2	15.3
D-Lactic acid produced, g/l, * (Boehringer Mannheim Kit)	8.7	0.6	7.9	8.2	0.7
Acetic acid produced, g/l, ** (Boehringer Mannheim Kit)	1.1	0	0	0	0
Starch	+	-	+	+	-

* The Boehringer Mannheim Kit (Cat. No. 139084) used to determine the concentration of L- and D-lactic acid in the growth medium consists of a) glycylglycine buffer, L-glutamic acid and stabilizers; b) β-nicotinamide-adenine dinucleotide (NAD); c) 1100 U of glutamate-pyruvate transaminase (GPT); and d) 3800 U of L-lactate dehydrogenase (for L-lactic acid) or D-lactate dehydrogenase (for D-lactic acid) (LDH), in separate containers.

The sample (0.1-10 ml) is pipetted into a cuvette (1 cm light path) containing a)+b)+c). The absorbance is read at 340 nm after which d) is added initiating the following reaction:

$$\text{1) L-lactate + NAD} \overset{\text{LDH}}{\leftrightarrow} \text{pyruvate + NADH + H}^{+}.$$

The amount of NADH formed in the above reaction is stoichiometric with the concentration of lactic acid. The absorbance is measured at 340 nm after completion of the reaction (about 10 minutes). The difference in absorbance before and after reaction is calculated and

compared to that of a blank (a cuvette not containing any sample). The amount of lactic acid in the sample is then calculated according to the following formulae:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \ [g/l]$$

where ΔA = absorbance difference of blank subtracted from that of sample,

V = final volume (ml),

v = sample volume (ml)

MW = molecular weight of the substance to be assayed,

d = light path (cm),

ϵ = absorption coefficient of NADH at 340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]

It follows for L-lactic acid

$$c = \frac{2.24 \times 90.1}{\epsilon \times 1 \times 0.1 \times 1000} \times \Delta A =$$

2.018 x $\Delta A/\epsilon$ [g L-lactic acid/l sample solution]

$$c = \frac{2.27 \times 90.1}{\epsilon \times 1 \times 0.1 \times 1000} \times \Delta A =$$

2.045 x $\Delta A/\epsilon$ [g D-lactic acid/l sample solution].

** Catalogue No. 148261.

Table 3

Fermentation patterns recorded in Bergey's Modified Medium

No.			SS28	SS129	SS131	SS151	SS258
1		Gram	+	+	+	+	+
2		Catalase	-	-	-	-	-
3		Oxydase	-	-	-	-	-
4		Motility	-	-	-	-	-
5		Spores	-	-	-	-	-
6		Strictly aerobic	-	-	-	-	-
7		Microaerophilic	+	+	+	+	+
8		Pigment	-	-	-	-	-
9		Growth, 15°C	-	-	-	-	-
10		Growth, 45°C	+	+	+	+	+
11		Starch hydrolysis	+	-	(-)	+	-
12		Arginine hydrolysis	-	-	-	-	-
13		Nitrate reduced	-	-	-	-	-
14		Gelatin liquefaction	-	-	-	-	-
15		Casein hydrolysis	-	-	-	-	-
16		Indole	-	-	-	-	-
17		H_2S	-	-	-	-	-
18		D-Lactic acid	+	+	+	+	+
19		L-Lactic acid	+	-	+	+	-
20		Amygdalin	+	-	+	+	-
21	L(+)	Arabinose	-	-	-	-	-
22		Cellobiose	+	-	+	+	-
23		Aesculin hydrolysis	+	-	+	+	-
24	D(-)	Fructose	+	+	+	+	+
25	D(+)	Galactose	+	+	+	+	+
26	D(+)	Glucose, acid	+	+	+	+	+
27		Glucose, gas	-	-	-	-	-
28		Gluconate	-	-	-	-	-
29		Lactose	+	+	+	+	+
30		Maltose	+	+	+	+	+
31	D(-)	Mannitol	-	+	-	-	+
32	D(+)	Mannose	+	+	+	+	+
33	D(+)	Melezitose	-	-	-	-	-
34	D(+)	Melibiose	+	+	+	-	+
35	D(+)	Raffinose	+	+	+	-	+
36	L(+)	Rhamnose	-	+	-	-	+
37		Ribose	-	-	-	-	-
38		Salicin	+	-	+	+	-
39	D(-)	Sorbitol	-	+	-	-	-
40		Sucrose	+	+	+	+	+
41		Trehalose	+	-	+	-	+
42	D(+)	Xylose	-	-	-	-	-

Identification

SS28 identified as *L. acidophilus/L. gasseri*.
100% accordance with *Bergey's Manual*.
No atypical reactions.

SS129 identified as *L. salivarius* subsp. *salivarius*.
87% accordance with *Bergey's Manual*.
Atypical reactions: mannose, trehalose, maltose, sorbitol.

SS131 identified as *L. acidophilus/L. gasseri*.
97% accordance with *Bergey's Manual*.
Atypical reactions: amygdalin.

SS151 identified as *L. crispatus*.
100% accordance with *Bergey's Manual*.
No atypical reactions.

SS258 identified as *L. salivarius* subsp. *salivarius*.
97% accordance with *Bergey's Manual*.
Atypical reactions: mannose.

EXAMPLE 2

Adhesion to squamous epithelial cells from *pars oesophagea*

A characteristic considered important for a probiotic strain is the ability to attach to the cells of the gastrointestinal tract. This attachment will enable the bacteria to multiply and colonize the stomach and the intestinal tract, thus contributing to maintaining a well-balanced microflora in the digestive tract.

It has been generally observed that the ability to attach to and colonize the surface of gastric epithelial cells is connected with host specificity (Lin, J.H.-C. and D.C. Savage, "Host specificity of the colonization of murine gastric epithelium by Lactobacilli", *FEMS Microbiol. Letters 24*, 1984, pp. 67-71; Tannock, G.W., O. Szylit, Y.

Duval and P. Raibau, "Colonization of tissue surfaces in the gastro-intestinal tract of gnotobiotic animals by Lactobacillus strains", *Can. J. Microbiol. 28*, 1982, pp. 1196-1198; Kotarski, S.F., Savage, D.C., "Models for study of the specificity by which indigenous Lactobacilli adhere to murine gastric epithelia", *Inf. and Immun. 26*, 1979, pp. 966-975; Fuller, R., "Ecological Studies of the Lactobacillus Flora Associated with the Crop Epithelium of the Fowl", *J. Appl. Bacteriol. 36*, 1973, pp. 131-139).

The five strains in question have been selected for their ability to attach to squamous epithelial cells of *pars oesophagea* from pig stomachs (Table 4).

Materials and Methods

The test is a slight modification of a test described by Fuller et al. (*Appl. and Environ. Microbiol. 35* (3), 1978).

Isolation of epithelial cells from pigs

Newborn piglets which had not yet been fed were killed by an intracardiac overdose of pentobarbitone. The stomach was opened by a slit along the greater curvature, and the oesophageal and gastric walls were gently washed with phosphate buffered saline (PBS), pH 7.3. Squamous epithelial cells from the *pars oesophagea* were brushed off into 2.5 ml of PBS and homogenized gently. This preparation can be stored frozen at -20°C.

Test for adhesion of bacteria to epithelial cells

The bacteria (all *Lactobacilli*) were grown overnight at 37°C in MRS medium (Oxoid CM 359). The test culture was diluted or centrifuged and resuspended in PBS, and the total count was adjusted by using a counting chamber to about $5x10^7$ to $1x10^8$ cells per ml. 0.2 ml of the epithelial cell suspension was mixed with 0.05 ml of bacterial suspension to give a ratio of approximately 100 bacteria to 1 epithelial cell. The mixture was rotated at 37°C for 30 minutes and examined for adhesion by phase-contrast microscopy. At least 5 cells were exam-

ined. Evaluation of the adhesion ability is described as: + = 4-15 bacteria, attached to the surface of 1 epithelial cell; ++ = 16-50 bacteria, attached to the surface of 1 epithelial cell, +++ = ≥ 50 bacteria, attached to the surface of 1 epithelial cell.

TABLE 4

Adhesion of bacteria to epithelial cells

Strain	*Adhesion*
SS28	+++
SS129	+++
SS131	+++
SS151	+
SS258	++

EXAMPLE 3

Acidification activity

In order to multiply in the intestinal tract, it is important that the bacteria can ferment the carbohydrates available in the partially decomposed feed, i.e. lactose from sowmilk and glucose, maltose and, to some extent, starch from the weaning feed.

One of the characteristics which is also considered important for a probiotic organism is the ability to produce lactic acid, thus reducing the pH, both in the stomach and in the upper part of the small intestine.

The five strains in question have been selected for acidification activity in media resembling partially decomposed feed.

The time it takes to reach the final pH is dependent on the growth conditions of the bacteria before the acidification test.

Materials and methods

Acidification activity in glucose

From an overnight MRS culture of the strain in question, 0.10 ml was inoculated into 10 ml of MRS broth (Oxoid CM 359) of pH 6.2. After incubation at 37°C for up to 48 hours in an atmosphere containing 10% CO_2 in H_2, the pH was measured.

Acidification activity in lactose

From an overnight MRS culture of the strain in question, 0.10 ml was inoculated into 10 ml of 9.5% reconstituted skim milk (SKM) which had been UHT treated and pasteurised. After incubation at 37°C for up to 48 hours in an atmosphere containing 10% CO_2 in H_2, the pH was measured.

Acidification activity in maltose/starch-containing medium

The medium (called MRS-P) was a modified MRS medium in which the glucose had been replaced by a mixture of maltose and soluble starch in equal amounts. The composition of the medium was as follows:

Bacteriological peptone	10.0	g
Bacto soytone	8.0	g
Yeast extract	4.0	g
Soluble starch	10.0	g
Maltose	10.0	g
Tween 80	1.0	ml
K_2HPO_4	2.0	g
Sodium acetate, $3H_2O$	5.0	g
Triammonium citrate	2.0	g
$MgSO_4$, $7H_2O$	0.2	g
$MnSO_4$, $4H_2O$	0.05	g
Demineralised water	ad 1000	ml

pH = 6.2

From an overnight MRS culture of the strain in question, 0.10 ml was inoculated into 10 ml of MRS-P. After incubation at 37°C for up to 48 hours in an atmosphere containing 10% CO_2 in H_2, the pH was measured.

Typical final pH values are shown in Table 5.

EXAMPLE 4

Bile tolerance

It is considered important for a probiotic organism to be resistant to the presence of bile in the digestive tract.

The amount of bile produced during 24 hours varies with the feeding time and the content of fat in the feed. In pigs the bile concentration is unlikely to exceed 0.9% in the contents of the small intestine. The strains in question have been selected for their tolerance to bile.

The bile tolerance was measured as the acidification activity in MRS (Oxoid CM 359) containing 0.9% of bile, added as 0.9 g of Bactooxgall (Difco B 128) per 1000 ml of broth.

Method

From an overnight-grown MRS broth of the strain in question, 0.10 ml was inoculated into 10 ml of MRS containing 0.9% of bile.

After incubation at 37°C for up to 48 hours in an atmosphere containing 10% CO_2 in H_2, the pH was measured.

Typical final values are shown in Table 5.

EXAMPLE 5

Acid tolerance

An important criterion for a probiotic strain is the ability to survive the passage of the stomach. The HCl production in a pig's stomach increases during the first period of the piglet's life until it is fully developed after approximately two months. The pH of the stomach varies greatly, depending on the nature and amount of the stomach contents. The pH is rarely less than 4.5 in a stomach containing feed, but pH in an empty stomach of a pig can be as low as 1.0-2.0.

The strains in question have been selected for their acid tolerance by testing the survival in a PBS medium of pH 2.5.

Materials and Methods

From a fully outgrown MRS culture, 0.10 ml was inoculated into 10 ml of phosphate buffered saline (PBS), pH 2.50 (adjusted with 0.1 N HCl).

Dilution series and plating on MRS was carried out at time 0 and 1 hour, and the log reduction was determined.

Typical reduction values are shown in Table 6.

TABLE 5

Acidification activities and bile tolerance
(incubation at 37°C for up to 48 hours)

Typical final pH values

Strain	*Medium* MRS	MRS + 0.9% of bile	MRS-P	SKM
SS28	3.86	4.21	4.02	4.93
SS129	3.72	4.55	4.11	4.43
SS131	3.89	3.92	4.01	5.07
SS151	3.74	3.88	3.75	3.82
SS258	3.69	4.57	4.10	4.36

TABLE 6

Acid tolerance (log CFU/ml reduction in PBS,
pH 2.5, 1 hour, 40°C)

Typical reduction values

Strain	Log CFU/ml reduction
SS28	3.23
SS129	0.67
SS131	1.92
SS151	0.63
SS258	0.59

EXAMPLE 6

Susceptibility to feed additives

The five selected strains have been tested for susceptibility to a number of growth promoters and antibiotics frequently used as feed additives. The susceptibility was measured as the minimum inhibitory concentration (MIC) values by inoculating the organisms into two-fold dilution series of the individual additives in an MRS broth. The bacterial concentration was approximately $5x10^5$/ml. The results are shown in Table 7.

TABLE 7

	MIC values (ppm)				
Feed additive	SS28	SS129	SS131	SS151	SS258
Avoparcin	<1.5	>100	25	100	>100
Olaquindox	>100	>100	>100	(>)100	>100
Carbadox	100	100	100	25	50
Flavofosfolipol	5	0.6	10	10	2.5
Nitrovin	31	>248	248	248	>248
Spiramycin	>200	>200	3.1	3.1	6.3
Tylosin	>62.5	>62.5	3.9	3.9	3.9
Virginiamycin	7.8	3.9	31	2.0	2.0
Zn-bacitracin	12.5	12.5	<1.6	12.5	12.5
$ZnCl_2$	250	1000	250	1000	1000

EXAMPLE 7

Susceptibility to feed additives present in pig feed

The five selected strains which were all rif^r were plated on solid MRS medium containing 50 µg/ml rifampicin and incubated anaerobically for 48 hours at 37°C. One colony of each strain was grown anaerobically in liquid MRS medium containing 50 µg/ml rifampicin for

18 hours at 37°C after which the strains were mixed in a ratio of 1:1:1:1:1 CFU/ml to a total of 4-10x10^8 CFU/ml.

25 g of a pig starter feed (Pri-mor from A/S Korn- og Foderstof Kompagniet, Denmark) were crushed and mixed with 75 ml of water. The feed additives were added immediately prior to the addition of 2.5 ml (1.6x10^7 CFU/ml) of the mixed bacterial cultures. The mixture was incubated with stirring for 4 hours at 37°C after which the pH was measured and dilution series were prepared. The incubated mixture was plated on solid MRS medium containing 50 µg/ml rifampicillin.

The results are shown in Table 8 in which the figures are the average of three experiments.

TABLE 8

Feed additive	Amount*	CFU/ml	pH
Avotan	40 ppm	7.4x10^7	5.68
Olaquindox	20 ppm	6.4x10^7	5.66
Carbadox	20 ppm	2.5x10^7	5.66
Flavomycin	25 ppm	5.3x10^7	5.69
Nitravin	30 ppm	2 x10^7	5.79
Spiramycin	80 ppm	1.8x10^7	5.84
Tylosin	40 ppm	3.6x10^7	5.82
Virginiamycin	50 ppm	1.3x10^7	5.83
Zn-bacitracin	80 ppm	6.5x10^6	5.79
$ZnCl_2$	100 mM	3.2x10^7	5.24
Control	0	7.6x10^7	5.61

* Maximum allowable dosage

It appears from Table 8 that the mixed bacterial cultures survive 4 hours of incubation in the feed containing the ten feed additives although bacterial growth is inhibited to a greater or lesser extent relative to the control.

EXAMPLE 8

H_2O_2 production

It is characteristic of some *Lactobacilli* to be able to produce H_2O_2 which may show an inhibitory effect against other microorganisms.

The five selected strains were tested for the ability to produce H_2O_2 by a method described by Marshall, V.M. (*J. Appl. Bacteriol. 47*, 1979, pp. 327-328), slightly modified with respect to the basic growth medium (MRS instead of acetate agar).

The reactions are shown in Table 9.

TABLE 9

Strain	H_2O_2 production
SS28	++
SS129	-
SS131	+
SS151	+
SS258	-

++ = strong production
+ = weak production
- = no production

EXAMPLE 9

Generation time

In order to be able to compete with the intestinal microflora, the probiotic lactic acid bacteria should have a brief generation time.

When grown in MRS medium at 40°C under anaerobic conditions, the selected strains showed the generation times indicated in Table 10.

TABLE 10

Strain	Generation time
SS28	62 minutes
SS129	40 minutes
SS131	50 minutes
SS151	47 minutes
SS258	34 minutes

EXAMPLE 10

Protective effect against experimentally induced *E. coli* diarrhoea in weaning pigs

The effect of the strains was investigated in weaning pigs challenged with *E. coli* 0149/K88.

28 pigs of both sexes from 3 litters, age 33-34 days, were removed from their mothers while still nursing and prior to exposure to solid feed. Each litter was divided into two groups, taking the weight and the sex of each animal into account in order to make the two groups as uniform as possible. All the pigs were ear-tagged, and faecal swab samples were taken from each animal.

One group from each litter was housed together as a treatment group, and the remaining three groups were housed together as the control group.

Both groups were housed in pens heavily contaminated with 0.5 l each of a fully outgrown hemolytic *E. coli* 0149/K88 towards which the pigs were known to be sensitive.

Each animal from the treatment group received an oral dose (by gavage) of a 10 ml mixture of equal amounts of overnight broths from the five strains = $2x10^9$/dose on arrival (day 1) and twice a day for two weeks. Faecal swab samples were taken from each animal in both groups every morning on days 1-16, 19 and 22.

Results: *E. coli* 0149/K88 was isolated from all animals at some time during the period, peaking on days 7-11.

From the control group, 7 out of 14 pigs died with the typical symptoms of colienteroxemia. The diagnosis was verified by autopsy, followed by bacteriology and serotyping of *E. coli* 0149/K88.

From the treated group, only 1 out of 14 pigs died, *E. coli* 0149/K88 being the primary cause.

χ^2 log rank = 5.95, $p < 0.015$

EXAMPLE 11

Effect on reducing the severity of experimentally induced *E. coli* diarrhoea in weaning pigs

In the experiment described in Example 10, all surviving pigs in the control group and in the group to which lactic acid bacteria were administered suffered from diarrhoea. In order to assess an effect on the severity of non-fatal cases, the body weights were recorded at the beginning of the experiment and after 14 days. The results are shown in Table 11.

TABLE 11

		Mean body weight, kg Day 0	Day 14	Average daily weight gain, g
Control group	7	9.16	10.60	101
Treatment group	13	8.45	11.70	231

The average daily weight gain of the *Lactobacillus* treated pigs was more than twice as high as that of the untreated group, indicating that the severity of the diarrhoeal outbreaks was reduced by the administration of the *Lactobacillus* cultures.

EXAMPLE 12

Field trial with a freeze-dried probiotic mixture

The field trial included 1260 newly weaned pigs from four herds, all suffering from weaning diarrhoea induced by *E. coli* 0149. 562 pigs were treated with a freeze-dried mixture of four probiotic strains (SS129, SS131, SS151 and SS258) which was administered as a "top dressing" in a dosage of 5×10^9 colony-forming units/pig/day for the first ten days of weaning. 437 pigs were treated conventionally with antibiotics, chemotherapeutic agens and/or a high dosage of zinc (positive control). The remaining 261 pigs did not receive any treatment (negative control). The results are shown in Table 12 below.

TABLE 12

Group	% days with diarrhoea	Mortality (%)
Non-treated	11.2	5.4
Zn-antibiotics	0.8	0
Probiotic bacteria	6.3	1.6

It appears from the Table that the frequence of diarrhoea was reduced by 50% by treatment with the lactic acid bacteria of the invention. Likewise, the mortality rate was considerably reduced. In herds with a low infection pressure, a positive effect of the bacteria in the form of an increased daily weight gain throughout the weaning period was also observed.

International Application No: PCT/ PCT/DK 88/00222

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line 31 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b
D-3300 Braunschweig
Federal Republic of Germany

Date of deposit [5]	Accession Number [5]
22 December 1987	DSM 4327

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

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MICROORGANISMS

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Address of depositary institution (including postal code and country) [4]

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Federal Republic of Germany

Date of deposit [5]	**Accession Number** [6]
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A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b
D-3300 Braunschweig
Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
22 December 1987	DSM 4326

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International Application No: PCT/ PCT/DK88/00222

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line 32 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b
D-3300 Braunschweig
Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
22 December 1987	DSM 4328

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. A lactic acid bacterium isolated from the gastrointestinal tract of a pig and selected according to the following criteria:

a) adhesion to pig gastrointestinal epithelial tissue after incubation of bacteria with pig epithelial cells in a ratio of 100:1 in phosphate buffered saline at 37°C for 30 minutes,

b) production of lactic acid as determined by

i) growth in MRS (Oxoid CM 359) medium, pH 6.2, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 4.0, or

ii) growth in 9.5% reconstituted skimmilk, pH 6.3-6.4, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 5.2, or

iii) growth in modified MRS medium without glucose, pH 6.2, containing equal amounts of maltose and soluble starch for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction to at least 4.5, or

iv) defined as the production of at least 10 g/l of lactic acid (D- and L-forms) when grown in MRS medium, pH 6.2, for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2,

c) bile tolerance as determined by growth in MRS medium, pH 6.2, supplemented with 0.9% bile for up to 48 hours at 37°C in an atmosphere containing 10% CO_2 in H_2, giving rise to a pH reduction comparable to the one defined in b) (i)-(iii),

d) acid tolerance as determined by a reduction in the number of colony-forming units/ml after incubation for 1 hour at 40°C and a pH of 2.5 not exceeding 3.2 log units, preferably less than 1 log unit,

e) a generation time of about 65 minutes, preferably 30-50 minutes, when grown in MRS medium at 40°C under anaerobic conditions,

or a functionally equivalent mutant thereof.

2. A bacterium according to claim 1, which adheres to pig gastrointestinal epithelial tissue in a number of at least 4, preferably at least 16, most preferably at least 50, bacteria per epithelial cell.

3. A bacterium according to claim 1, which is able to produce an antimicrobial agent such as hydrogen peroxide.

4. A bacterium according to claim 1, wherein the strain is selected from the group consisting of: *L. acidophilus/L. gasseri* isolated from the duodenum of a suckling pig; *L. salivarius* subsp. *salivarius* isolated from the jejunum of a weaning pig; *L. acidophilus/L. gasseri* isolated from the ileum of a weaning pig; *L. crispatus* isolated from the jejunum of a suckling pig; and *L. salivarius* subsp. *salivarius* isolated from the *pars oesophagea* of a suckling pig.

5. A bacterium according to claim 4 which is selected from the group consisting of:

L. acidophilus/L. gasseri SS28	DSM 4324
L. salivarius subsp. *salivarius* SS129	DSM 4325
L. acidophilus/L. gasseri SS131	DSM 4326
L. crispatus SS151	DSM 4327
L. salivarius subsp. *salivarius* SS258	DSM 4328

6. A bacterium according to claim 5, which is *L. salivarius* subsp. *salivarius* SS129, DSM 4325, or *L. salivarius* subsp. *salivarius* SS258, DSM 4328.

7. A liquid or frozen concentrate comprising a lactic acid bacterium according to any of claims 1-6.

8. A concentrate according to claim 7, which comprises $1x10^4$-$1x10^{12}$ viable colony-forming units/ml.

9. A concentrate according to claim 7, which comprises a mixture of two or more *Lactobacillus* strains according to any of claims 4-6.

10. A freeze-dried or spray-dried powder comprising a lactic acid bacterium according to any of claims 1-6.

11. A powder according to claim 10 which comprises 10^4-10^{12} viable colony-forming units/g.

12. A powder according to claim 10 which comprises a mixture of two or more *Lactobacillus* strains according to any of claims 4-6.

13. A fermented milk product, which comprises a lactic acid bacterium according to any of claims 1-6.

14. A fermented milk product according to claim 13, which additionally comprises *L. acidophilus* and/or *Bifidobacterium* spp.

15. A fermented milk product according to claim 13 or 14, which comprises a mixture of two or more *Lactobacillus* strains according to any of claims 4-6.

16. A veterinary composition for the prophylaxis or treatment of gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms, the composition comprising a lactic acid bacterium according to any of claims 1-6 and an excipient or carrier.

17. A composition according to claim 16 which comprises 10^3-10^{12} viable colony-forming units/g.

18. A composition according to claim 16 which is formulated as a powder, granulate, tablet, capsule, paste, gel, drench, mixture or suspension.

19. A composition according to claim 18, wherein the powder is the powder according to any of claims 10-12.

20. A composition according to claim 18 or 19, wherein the powder, granulate, tablet or capsule is provided with a coating which is degradable in a gastrointestinal environment.

21. A composition according to claim 20, wherein the coating is one which is soluble in the stomach or in the upper part of the small intestine.

22. A composition according to claim 18, wherein the suspension is a suspension of the powder of any of claims 10-12 in an aqueous or oily medium, such as in the drinking water of the pig, an electrolyte solution or in a reconstituted sowmilk replacer.

23. A composition according to any of claims 16-22 which comprises a mixture of two or more *Lactobacillus* strains according to any of claims 4-6.

24. A process of producing a lactic acid bacterium according to claim 1, the process comprising isolating and selecting a lactic acid bacterial strain in accordance with the criteria defined in claim 1, growing the so selected bacterium in a suitable medium under anaerobic conditions for a period of time sufficient to provide at least 10^6 viable lactic acid bacteria/ml of medium, and harvesting the resulting bacteria from the medium.

25. Use of a lactic acid bacterium according to any of claims 1-6 for the production of a fermented milk product.

26. Use of a liquid or frozen concentrate according to any of claims 7-9 for the production of a fermented milk product.

27. Use of a freeze-dried or spray-dried powder according to any of claims 10-12 for the production of a fermented milk product.

28. Use of a lactic acid bacterium according to any of claims 1-6 for preparing a veterinary composition for the prophylaxis or treatment of gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms.

29. Use according to claim 28, wherein the pigs for which the composition is intended are suckling or weaning pigs or porkers.

30. Use according to claim 28, wherein the composition is adapted to be sprayed or sprinkled in the environment of the pigs.

31. Use according to claim 30, wherein the composition is in the form of a powder according to any of claims 10-12.

32. Use according to claim 28, wherein the composition is in the form of a suspension in an aqueous or oily medium, such as the drinking water of the pigs, an electrolyte solution or a reconstituted sowmilk replacer.

33. Use according to claim 28, wherein the composition is in a form suited for administration to a sow.

34. Use according to claim 28, wherein the composition is adapted to be mixed with the feed of weaning pigs or porkers.

35. Use according to claim 34, wherein the composition is in the form of a powder according to any of claims 10-12.

36. Use according to any of claims 32-35, wherein the amount of lactic acid bacteria per dosage of the composition is in the range of 10^4-10^{12}, preferably 10^6-10^{10}, colony-forming units.

37. A method of preventing or treating gastrointestinal infections in pigs caused by enteropathogenic microorganisms, the method comprising administering a therapeutically effective dosage of a lactic acid bacterium according to any of claims 1-6 to pigs.

38. A method according to claim 37, wherein the pigs are suckling or weaning pigs or porkers.

39. A method according to claim 37, wherein the lactic acid bacterium is administered as a powder according to any of claims 10-12, the powder being sprayed or sprinkled in the environment of the pigs.

40. A method according to claim 37, wherein the lactic acid bacteria are administered in the form of a suspension thereof in an aqueous or oily medium, such as the drinking water of the pigs, an electrolyte solution or a reconstituted sowmilk replacer.

41. A method according to claim 40, wherein the amount of lactic acid bacteria is in the range of 10^4-10^{12}, preferably 10^6-10^{10}, viable colony-forming units per dosage.

42. A method according to claim 41, wherein the lactic acid bacteria are administered 1-3 times a day, for a period of up to 14 days.

43. A method according to claim 37, wherein the lactic acid bacteria are administered in the form of a powder according to any of claims 10-12, the powder being admixed with the feed of the pigs.

44. A method according to claim 43, wherein the amount of lactic acid bacteria administered is in the range of 10^4-10^{12}, preferably 10^6-10^{10}, viable colony-forming units per dosage.

45. A method according to claim 44, wherein the lactic acid bacteria are administered 1-3 times a day, for a period of up to 14 days.

46. A method of preventing or treating gastrointestinal tract infections in pigs caused by enteropathogenic microorganisms, the method comprising administering to a gestating or lactating sow an amount of lactic acid bacteria according to any of claims 1-6 which is sufficient to provide a therapeutically effective amount of the bacteria in the environment of the sow.

47. A method according to claim 46, wherein the amount of lactic acid bacteria administered is in the range of 10^5-10^{12}, preferably 10^7-10^{11}, viable colony-forming units per dosage.

48. A method according to claim 47, wherein the lactic acid bacteria are administered 1-3 times a day for a period of up to 28 days.

49. A method according to any of claims 46-48, wherein the lactic acid bacteria are administered in the form of a powder according to any of claims 10-12.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00222

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

C 12 N 1/20, A 61 K 35/74 // C 12 R 1:225

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC 4	C 12 N 1/20; A 61 K 35/74; A 61 K 39/07
US Cl	424:93; 426:2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above. Data base search: WPI,WPIL, CA, BIOSIS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	EP, A3, 0 203 586 (PIONEER HI-BRED INTERNATIONAL) 3 December 1986 see page 6, line 16-21 & JP, 62089625	1-36
Y	Journal of Applied Bacteriology, 1-39, 1980, Vol 48, Barrow et al "The Attachment of Bacteria to the Gastric Epithelium of the Pig and its Importance in the Microecology of the Intestine p 147-154, see p 150, line 33-40	1-36
Y	EP, A3, 0 199 535 (THE NEW ENGLAND MEDICAL CENTER HOSPITALS INC) 29 October 1986 & JP, 61280433	1-36
Y	EP, A2, 0 033 584 (NURMI ESKO VILJO et al) 12 August 1981 & CA, 1151066 AT,E, 7201 US, 4689226	1-36

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1989-03-06	1989 -03- 28
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,Y	EP, A2, 0 271 364 (BIOREM C.C.) 15 June 1988 see claim 1 &	1-36

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [x] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [x] Claim numbers 37-49 because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.